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An analysis of 14 molecular markers for monitoring osteoarthritis: segregation of the markers into clusters and distinguishing osteoarthritis at baseline

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Summary

Objective: To investigate the relationships between serum and urinary molecular markers (MM) used to monitor osteoarthritis.

Design: Forty osteoarthritis patients had blood and urine collected at baseline and 1, 3, 6 and 12 months later. Specimens from 20 controls were obtained twice at a one month interval. The concentration of 14 different markers was determined at each time point and the data were analyzed by statistical methodology.

Results: The markers could be divided by the method of principal components analysis into five clusters of related markers: inflammation markers (C-reactive protein, tumor necrosis receptor type I and tumor necrosis receptor type II, interleukin 6, eosinophilic cationic protein), bone markers (bone sialoprotein, hydroxylysyl pyridinoline, lysyl pyridinoline), putative markers of cartilage anabolism (carboxypropeptide of type II procollagen, hyaluronan, epitope 846) and catabolism (keratan sulfate, cartilage oligomeric matrix protein), and transforming growth factor beta. Three markers (tumor necrosis factor receptor II, cartilage oligomeric matrix protein and epitope 846) from independent clusters discriminated osteoarthritis patients from controls. Inflammation was not a confounding factor in measurement, but a recognizable distinguishing factor in osteoarthritis.

Conclusions: The markers separated into rational groups on the basis of their covariance, a finding with independent biochemical support. The covariance of markers from the same cluster suggests the use of a representative marker from the cluster to reflect changes in osteoarthritis. If multiple markers are being measured within a single cluster, then the use of a weighted cluster 'factor' may be preferable to the separate use of individual markers. © 2000 OsteoArthritis Research Society International

Key words: Molecular markers, Osteoarthritis, CRP, Hyaluronan, Pyridinoline.

Introduction

Osteoarthritis (OA) of the knee occurs with increasing frequency and severity with age. It is generally characterized by joint space narrowing on X-ray, knee pain, and a loss of joint function. Except for OA arising from injury to the knee, it is often difficult to clearly distinguish the clinical disease from a slow deterioration of the cartilage with age. Even though this is a disease afflicting a significant portion of the population, its evolution over decades has made clinical studies difficult. Changes in disease status over a single year are often small and difficult to quantitate. Under such circumstances, the identification, if possible, of molecular markers in easily sampled body fluids such as blood or urine, which reflect differences in disease progression rates, would greatly facilitate clinical studies.[1] A good molecular marker should define differences between OA patients and normal individuals. It should also define changes in patient clinical status over time.

A number of candidate molecular markers (MM) have been identified.[2] The MM that have so far been examined can be classified into two types, skeletal and inflammation markers. Skeletal markers are related to changes in cartilage or bone metabolism: keratan sulfate (KS),[3–5] the carboxypropeptide of type II procollagen (CPII),[6,7] bone sialoprotein (BSP),[8,9] cartilage oligomeric matrix protein (COMP),[7,9,10] aggrecan fetal epitope (epitope 846),[7,11,12] hydroxylysyl pyridinoline (HP) and lysyl pyridinoline (LP).[13–15] Disease markers are related to inflammation: C-reactive protein (CRP),[16] tumor necrosis factor receptor type I (TNF-RI) and tumor necrosis factor receptor type II (TNF-RII), interleukin 6 (IL-6),[17] hyaluronan (HA)[18–20] transforming growth factor beta (TGFβ), and eosinophil cationic protein (ECP).[21–23]

Molecular markers of skeletal metabolism and inflammation from easily sampled body fluids were examined for differentiating individuals with OA from control individuals. Markers that have sufficient discriminatory power at baseline to distinguish between OA patients and normal individuals are candidates for monitoring changes in disease status. In addition, statistical methodology was applied to

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examine the interrelationships among markers in order to group markers into related clusters. This allows the determination of which markers give independent information and which markers should be grouped together as related markers. Such a comprehensive analysis has never previously been attempted.

Patients and methods

PATIENTS

Sera and 24 h urines were collected from 34 female and five male patients with a diagnosis of idiopathic OA and involvement of at least one large joint (knee or hip) of radiological grade 1–3 by ACR OA criteria.[24,25] Their ages ranged from 45 to 61 with a mean age of 57 years. Each patient was removed from prior therapy (NSAID) for one week prior to the baseline visit. Five mls of blood and a 24 h urine specimen were taken at baseline. After the baseline visit, patients were placed on piroxicam 20 mg/day, and blood and urine specimens collected at visits 1, 3, 6, and 12 months later. A control cohort of 13 females and eight males (N) who were without joint pain (age range 45 to 63 with a mean of 50 years) were sampled twice at a one month interval. Blood as serum and urine were aliquoted and stored at −72°C until assayed. All samples for each assay were measured at one time in order to minimize variability.

ASSAYS

KS was measured using antibody AN9P1 as the $F(ab')_2$ in a competitive radioimmunoassay (RIA).[5] CP-II was also measured by RIA;[7] BSP was measured by ELISA;[8] COMP was measured using rabbit polyclonal antibody;[10] epitope 846 was measured as described.[5] Commercial ELISAs were used to measure CRP (Hemagen CRP Kit, Hemagen Diagnostics, Inc., Waltham, MA); TNF-RI (Quantikine™ Human sTNF RI Immunoassay), TNF-RII (Quantikine™ Human sTNF RII Immunoassay), IL-6 (High Sensitivity Human IL-6 Immunoassay) and TGFβ1 (Quantikine™ Human TGFβ1 Immunoassay) (R&D Systems, Minneapolis, MN); ECP and HA (Pharmacia Diagnostics AB, Uppsala, Sweden).

Hydroxylysyl pyridinoline (HP) and lysyl pyridinoline (LP) were analyzed after hydrolysis of urinary protein and separation of the cross-links on HPLC using a fluorescence detector.[13] Creatinine was determined for each urine on a clinical Boehringer Mannheim Hitachi 717 analyzer using a modified Jaffe reaction and the values for HP and LP divided by the creatinine concentration.

STATISTICAL PROCODURES

Statistical analyses were carried out with software from SAS (SAS Institute, Cary, NC 27512) and Statistica (StatSoft Inc., 2325 E. 13th St., Tulsa, OK 74104). The control and osteoarthritis groups were compared at baseline by Wilcoxon rank sum statistics, and by calculation of geometric means and 95% population confidence bounds. For all statistical tests that require normal distributions, the use of logarithms of all independent variables except age was necessary. Possible associations in data at baseline were explored by SAS Proc Factor, using principal components analysis with orthomax factor rotation and by Spearman rank order correlation.

Results

DIFFERENCES AMONG MOLECULAR MARKERS, OA PATIENTS VS. HEALTHY INDIVIDUALS

In order to learn whether any of the MM are suitable for monitoring disease status in OA clinical trials, it was first determined which of the MM were statistically different between OA patients and healthy controls. The baseline visits of the control population were compared with the baseline visits of the OA patients taken after a one week washout without drug. The data for each of the 14 MM are summarized in Table I. The values for the MM generally were not normally distributed, and *it was necessary to transform the data to logarithms to obtain approximately normal distributions.* Therefore, geometric means were determined, and 95% ranges rather than standard deviations were calculated. The values were compared using Wilcoxon Rank Sum Scores, which do not depend on distributions.

BSP, hyaluronan, C-propeptide of type II collagen, and TGFβ1 all failed to distinguish the OA from the control group. The ten other MM (TNF-RI, TNF-RII, CRP, ECP, IL-6, KS, HP/Cre, LP/Cre, COMP, epitope 846) showed highly significant differences between controls and OA patients. In all cases, the range of the control and the OA populations overlapped.

DISCRIMINANT ANALYSIS

Discriminant analysis was used to further investigate which of the markers (plus age), singly or in combination, were best able to distinguish between OA patients and control individuals. Preliminary results have been reported.[26] Stepwise backward elimination of non-informative variables, with cross-validation at each step, was used to arrive at an optimal linear model. Linear and kernel-based discriminant methods both yielded comparable results. The final model contained TNF RII, COMP, epitope 846, and age as significant ($P \leq 0.01$) discriminating variables. However, use of TNF RII alone provided reasonably good discrimination, with correct classification of 80% of controls and 86% of OA patients. In the factor analysis (see below), TNF RII, COMP and epitope 846 were not correlated with each other.

LOGISTIC REGRESSION

To aid in understanding the ability of markers to distinguish between OA patients and control individuals, logistic regression was carried out, with disease status (0 or 1) as the dependent variable, and TNF RII and COMP as the predictor variables. Because of the limitation of the graphical display to two dimensions, epitope 846 was not included. In the graphical display (Fig. 1), each point represents one patient, either OA or control, plotted according to the values of the predictor variables. Existence of OA is likely ($P \geq 0.90$) in the upper region of the graph, and is unlikely ($P \leq 0.10$) in the lower region. The contours represent equal ($P = 0.10$) intervals of predicted disease status in the logistic model. The effectiveness of the

Table I
*Comparison of molecular markers between osteoarthritis patients and normal individuals**

Marker†	Units	Normal geometric mean	Confidence limits 95%	*N*	OA geometric mean	Confidence limits 95%	*N*	N vs OA *P*-value
BSP	ng/ml	98.7	51.7–188	21	93.3	44.7–195	34	0.8016
IL-6	pg/ml	1.67	0.51–5.46	21	2.99	0.75–11.9	39	0.0043
Epitope 846	μg/ml	0.087	0.027–0.28	21	0.05	0.016–0.156	36	0.0006
KS	μg/ml	2.18	1.02–4.66	21	1.55	0.88–2.75	36	0.0008
CPII	ng/ml	24.01	18.9–30.4	21	22.95	18.5–28.5	37	0.1857
HA	ng/ml	43.24	18.5–101	21	49.85	10.2–244	37	0.2240
ECP	ng/ml	9.13	3.28–25.4	21	13.83	4.64–41.3	36	0.0089
CRP	μg/ml	1.64	0.34–7.97	20	5.03	0.58–43.8	39	0.0004
COMP	μg/ml	10.72	7.19–16.0	21	8.84	5.45–14.3	39	0.0011
HP/Cre	nM/mM‡	31.4	22.1–44.5	21	43.3	23.4–79.8	37	0.0001
LP/Cre	nM/mM	10.5	6.0–18.4	21	14.0	5.88–33.4	37	0.0040
TNF RI	pg/ml	995	568–1745	20	1449	834–2517	37	0.0001
TNF RII	pg/ml	1569	897–2747	20	2463	1424–4261	37	0.0001
TGFβ1	ng/ml	47.0	30.1–73.5	20	41.5	23.8–72.3	36	0.1440

*For each variable, the $\log_{10}$ of the parameter was taken to convert the numbers into a normal distribution. Statistical significance between OA and normal populations was determined using the Wilcoxon Rank test. Geometric means and confidence bounds for 95% of the population are given rather than standard deviations because of the lack of normality of the untransformed variables.

†Marker abbreviations: BSP, bone sialoprotein; IL-6, interleukin 6; epitope 846, aggrecan fetal epitope 846;.KS, keratan sulfate; CPII, C-propeptide of type II collagen; HA, hyaluronan; ECP, eosinophil cationic protein; CRP, C-reactive protein; COMP, cartilage oligomeric protein; HP/Cre, hydroxylysyl pyridinoline/creatinine; LP/Cre, lysyl pyridinoline/creatinine; TNF RI; tumor necrosis receptor type I; TNF R2, tumor necrosis factor receptor type II; TGFβ1, transforming growth factor beta_1.

discrimination of OA and the control population can be readily observed in the display.

PRINCIPAL COMPONENTS ANALYSIS

Factor analysis by the method of principal components was carried out to determine which MM were significantly correlated with one another. Disease, except insofar as it contributes to a broader range of values for the MM, was not involved in this determination, and both control and OA individuals were grouped together. Age of subject was also included as a variable. The five principal components that account for 72% of the variance summarize the relationships. The principal components matrix was rotated by the orthomax method, and the loading coefficients of each of the 15 variables in each of the five principal components are reported (Table II). The primary contributors to each principal component are indicated in bold-face. Although only the results at baseline are reported, the same marker associations and groupings in clusters were found at all time points, i.e., baseline, 1, 3, 6 and 12 months.

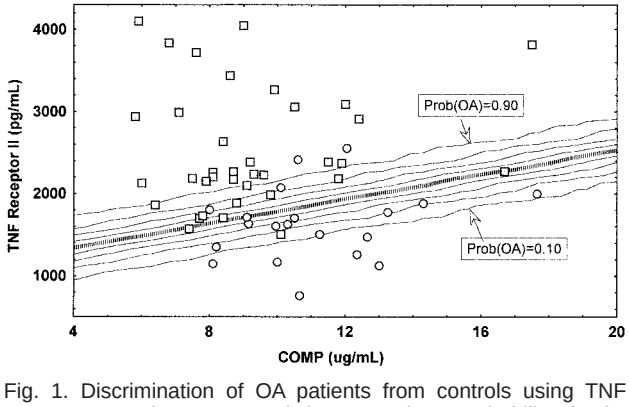

Fig. 1. Discrimination of OA patients from controls using TNF receptor II and COMP. Logistic regression: Probability {OA}= exp{A+B*X+C*Y}/(1+exp{A+B*X+C*Y}), where A=−6.372, B=−0.456, X=COMP, C=0.00613, Y=TNF RII and the contour lines=intervals of *P*=0.10 of predicted OA in the logistic model. ○=Normal, □=OA.

Factor 1 accounted for the largest portion of the variance and was comprised of markers of inflammation (IL-6, CRP, TNF RI, TNF RII, and ECP). There was an association of less significance between increasing values for the inflammation markers and increasing age. This latter association has been previously reported for CRP in the general population.

Factor 2 was comprised of markers considered to be primarily of bone origin (HP, LP and BSP), although a portion of HP also arises from cartilage.

Four of the remaining six MM are products of cartilage (epitope 846, CPII, COMP, KS) and they divided into two factors, Factor 3 (epitope 846, HA, CPII), and Factor 4 (COMP, KS). HA, although found in cartilage, is far more abundant in synovial fluid, yet it aligned with Factor 3. TGFβ1 is a growth factor without any known relationship to any of the other MM. It is not surprising that it segregated into a separate factor, Factor 5.

Discussion

Fourteen MM that might be related to the status or progression of OA were measured. Typically, when we compared our values to those in the literature, there was close agreement with values for the control population and the OA population only when the same measurement methodology was used. The largest disagreements were in cases where different monoclonal antibodies or, as importantly, different standards were used as the basis for

Table II
Principal component analysis coefficients isolation of independent factors†*

Molecular marker‡	Factor 1§	Factor 2	Factor 3	Factor 4	Factor 5
IL6	**0.879**	−0.003	−0.092	0.001	0.057
CRP	**0.802**	0.270	−0.114	−0.072	0.087
TNFRI	**0.795**	0.306	0.067	−0.106	−0.184
TNFRII	**0.748**	0.344	0.151	−0.057	−0.312
ECP	**0.663**	−0.186	0.133	−0.134	0.163
LP/Cre	0.138	**0.928**	0.080	0.003	−0.047
HP/Cre	0.290	**0.903**	0.007	−0.135	−0.075
BSP	−0.232	**0.531**	−0.125	0.415	0.250
Epitope 846	−0.159	−0.131	**0.711**	0.298	0.050
HA	0.273	0.370	**0.646**	−0.032	−0.092
Age	0.462	0.078	**0.606**	−0.307	−0.147
CPII	−0.223	−0.093	**0.568**	0.124	0.446
COMP	0.092	0.032	0.058	**0.846**	0.194
KS	−0.336	−0.103	0.157	**0.704**	−0.196
TGFβ1	0.076	−0.005	0.011	0.045	**0.882**
Variance due to Factor¶	3.69	2.46	1.73	1.64	1.32

*Values from normal and OA individuals were utilized to determine the correlation matrix. Disease was not considered a variable.

†Matrix rotation and determination of the number of factors were carried out in SAS with the orthomax software and the requirement that the eigenvalues must be greater than one for significance. Five factors were found.

‡The abbreviations for each marker are given in Table I.

§The primary components in each Factor are shown in bold.

¶The five factors together account for 72% of the total variance.

the assay, for example, the keratan sulfate measurements using monoclonal antibody AN9P1 or 7D4 or the TNF Type II receptor using the R & D or the Medgenix kits. In one case, IL-6, our controls suggested that our lower values were a more accurate measure. However, in other cases, there were no simple explanations; even measurements made in the same laboratory with the same methodology can differ in different studies. The differences were greater with ELISA methods than with biochemical methods (HP, LP). In addition to differences in the study population, the extent of color development with a particular set of reagents and the lack of universal standards may contribute to these literature differences. For these reasons, all samples were assayed at the same time with the same set of reagents. This includes not only the baseline control and OA specimens reported here, but all patient visits. While this does not eliminate inter-laboratory differences, it minimizes variability within the study.

Age was included as an independent variable in all analyses, in order to capture possible interactions between age and the markers in distinguishing between control and OA subjects. Even though the control and OA populations were not perfectly age-matched, this approach was considered better than collecting an additional set of controls and determining values in a separate set of measurements. Only two of the 14 markers were found to have a significant age dependency when regressed on age and disease: epitope 846 (P=0.02) and HA (P=0.006).

Among the MM, the inflammatory MM gave the largest separation of OA and control individuals. Yet none of the inflammatory MM showed changes of comparable magnitude to RA. For example, in the control population CRP is around 1 μg/mL. In the OA population, CRP is elevated to around 5 μg/mL. Sharif *et al*.[27] have published comparable findings. In RA, the CRP value may be elevated from 20 to 100 μg/mL or more, and it has been found to be a useful measure of disease activity and therapy.[16] Moreover, the inflammatory MM are nonspecific measures of disease change; they are elevated in many diseases. Thus their elevation becomes significant only when a primary diagnosis of OA is made and other possible confounding clinical conditions have been excluded.

The most statistically significant elevation was found for TNF-RII. It along with COMP, epitope 846 and age permit placement of approximately 90% of both the OA and the controls into the correct diagnosis. Thus the best discrimination was found when one marker from each of three independent clusters was used. However, in spite of statistically significant differences in skeletal markers between the group of OA patients and the group of controls, the overlap of patients and controls thwarts the utility of a single skeletal marker to reliably categorize individuals.

The failure to find a significant difference in HA between normal and OA is dependent on our choice of statistical methodology. Because our HA values did not show a normal distribution, a log transformation of the variables was carried out before statistical tests were applied. If we had used a standard *t*-test on the arithmetic means, controls=42.8 vs. OA=66.8 (a statistically invalid procedure with our data set), we too would have found, like the literature, a significant difference for HA between control and OA. Moreover, the highly significant age dependency of HA values must be taken into account in any analysis of HA for monitoring OA.

The main role for principal components analysis in experimental science is for hypothesis generation. By computing the complete correlation matrix, secondary correlations that occur when multiple correlations are done pairwise are removed. By determining which MM are associated (independent of disease), mechanistic linkages can be suggested. With the exceptions of HA, our analysis sorted the MM into independent factors consistent with known biochemical relationships. Thus the clustering of the inflammation MM together has a strong mechanistic basis. The correlation between IL-6 and CRP in man is strong,[16] anti-IL-6 antibodies dramatically lower CRP levels,[28,29] and

IL-6 elevation has been correlated with TNF.[30] The exocytosis of ECP by eosinophils has been taken as a measure of inflammation in rheumatoid arthritis.[22,23] The shedding of TNF R occurs during exposure to IL-1, TNF, PDGF and IGF1.[31,32]

The most interesting outcome of principal components analysis was the segregation of structural MM into factors 2, 3 and 4. This segregation appears to have a biochemical basis: factor 2 is associated with bone markers, factor 3 is associated with putative synthetic markers of cartilage assembly, and factor 4 is associated with putative markers of cartilage degradation. Thus in this case, the association of MM into factors seems to be a valid hypothesis generation process.

Because we did not X-ray our control population, it is possible that some of the controls may have had asymptomatic OA. Such an occurrence would not affect the outcome of our factor analysis. It depends only on whether markers change in tandem or not—controls and OA patients are lumped together in the analysis. It could, however, potentially cause us to underestimate the ability of skeletal markers to differentiate between controls and OA patients.

The principal components of factor 2, BSP, LP and HP, arise principally from bone, although HP also has a cartilage component in it. We used two statistical approaches to isolate the cartilage component and found by both that cartilage component of HP was stably associated with the inflammation markers (Appendix). This leads to the hypothesis that crosslink loss from cartilage is related to the degree of inflammation in OA; it is only weakly associated with changes in degradation markers that are based on normal cartilage turnover.

The concentrations in blood of TGFβ1 were uncorrelated with any of the other MM. This implies that if TGFβ1 plays a role in OA, it does not do so in a way that leads to an association with either the skeletal or inflammation markers we measured.

Conclusions

Our results confirm that inflammation in OA is readily measurable. Markers such as CRP and TNF type II receptor are elevated although much more modestly than in RA or other inflammatory diseases. The statistical independence of the bone, cartilage catabolism and anabolism, and TGFβ1 clusters from the inflammation cluster suggests that changes in these markers are independent of inflammation. The magnitude of change in the skeletal markers was disappointing in these cross-sectional patients. It augurs poorly for their utility in the clinic and suggests that better markers need to be found. Finally, the segregation of markers into different independent clusters suggests that clinical correlations should be carried out using the independent clusters rather than the individual covariant markers. However, the use of a single representative marker from each cluster is more efficient and economical than making multiple redundant measurements. New markers should be examined to determine their correlation with existing markers. If they belong to a cluster of molecules that gives essentially the same information, they are potentially redundant for disease monitoring.

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Appendix

It was of interest to isolate the cartilage contribution from the bone contribution in HP. The very high rate of bone loss in Paget's disease should give an estimate of the ratio of HP and LP from bone. Contributions of extra-osseous tissue including cartilage to HP (eHP) could in principle be obtained from total HP by subtraction of LP times a factor (α) whose value is the ratio of HP to LP due to bone catabolism alone. Thus eHP can be estimated as:

$$\text{eHP/Creatinine} = (\text{HP} - \alpha * \text{LP})/\text{Creatinine}$$

In Paget's disease, the HP/LP ratio is approximately 2.4.[33] Thus eHP was estimated using α=2.4 and the principal components analysis was repeated with eHP/Cre instead of HP/Cre. eHP is associated with markers of inflammation, not bone metabolism. The composition of the other factors remained essentially unchanged, although the factor containing the remaining markers of bone metabolism, LP and BSP, assumed less importance than in the first analysis.

The value of α in the calculation of eHP was estimated independently, based on the factor analysis alone. Trial values of α from 0.0 to 3.0, in steps of 0.1, were used to calculate eHP, the factor analysis was repeated, and loading coefficients for eHP in the inflammation (IL-6, etc.) and bone (LP, BSP) principal components were examined. As α approached 2.5, the correlation of eHP with bone markers approached zero, and the loading coefficient of eHP reached a maximum of 0.63 in its association with inflammation markers. Values of α either smaller or larger than 2.5 decreased the independence of the separation. To further confirm that eHP was correlated with the inflammation markers, the correlation of CRP and eHP was determined. eHP and CRP show a highly significant correlation (P=0.01).